Transcription factor Gbx2 acts cell-nonautonomously to regulate the formation of lineage-restriction boundaries of the thalamus

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Relatively little is known about the development of the thalamus, especially its differentiation into distinct nuclei. We demonstrate here that Gbx2-expressing cells in mouse diencephalon contribute to the entire thalamic nuclear complex. However, the neuronal precursors for different thalamic nuclei display temporally distinct Gbx2 expression patterns. Gbx2-expressing cells and their descendents form sharp lineage-restriction boundaries delineating the thalamus from the pretectum, epithalamus and prethalamus, revealing multiple compartmental boundaries within the mouse diencephalon. Without Gbx2, cells originating from the thalamus abnormally contribute to the epithalamus and pretectum. This abnormality does not result from an overt defect in patterning or cell-fate specification in Gbx2 mutants. Chimeric and genetic mosaic analysis demonstrate that Gbx2 plays a cell-nonautonomous role in controlling segregation of postmitotic thalamic neurons from the neighboring brain structures that do not express Gbx2. We propose that, within the developing thalamus, the dynamic and differential expression of Gbx2 may be involved in the specific segregation of thalamic neurons, leading to partition of the thalamus into different nuclei.

KEY WORDS: Thalamus, Compartment, Lineage restriction, Fate map, Mouse, Transcription factor, Gbx2

INTRODUCTION

The thalamus in mammals is composed of dozens of nuclei, which are aggregates of neurons, and each nucleus displays unique cytoarchitecture and function (Jones, 2007). Some thalamic nuclei project topographically to specific areas of the cortex, having a primary role in processing and relaying sensory input from the periphery to the cortex; other nuclei broadly project to the cortex, regulating the states of consciousness of the cortex (Jones, 2001; Jones, 2007). The thalamus develops from the diencephalic part of the neural tube. Based on morphology and gene expression, the vertebrate diencephalon is transiently divided into three transverse segments called prosomeres 1-3 (p1-3), which are believed to give rise to the pretectum, epithalamus and thalamus, and prethalamus, respectively (Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). Virtually all thalamic neurons in mice are generated from the alar plate of the p2 segment between embryonic day (E) 10.5 and E16.5 (Angelvise, 1970). Between E14.5 and E18.5, the diencephalon is progressively partitioned into discrete neuronal groups that signify their differentiation into nuclei (Jones, 2007). Little is known about the mechanism governing the selective segregation of postmitotic neurons to form the thalamic nuclear complex.

The p2 segment of the diencephalon is defined by the expression of a homeobox gene, Gbx2, in the mouse embryo (Bouillet et al., 1995; Bulfone et al., 1993; Miyashita-Lin et al., 1999; Nakagawa and O'Leary, 2001). In both mouse and monkey, Gbx2 expression is restricted to a subset of thalamic nuclei at birth and in adulthood (Jones and Rubenstein, 2004). Deletion of Gbx2 in mice leads to an almost complete loss of axonal connections between the cortex and the thalamus (Hevner et al., 2002; Miyashita-Lin et al., 1999). In addition, Gbx2-deficient mice exhibit severe defects in histogenesis of the thalamus and loss of a subset of thalamic nuclei, suggesting that Gbx2 may play an important role in differentiation of thalamic nuclei (Miyashita-Lin et al., 1999; Nakagawa and O'Leary, 2001). However, the molecular and cellular basis of the thalamic defects due to the mutation of Gbx2 is largely unknown.

The present study examines the development of the thalamus by analyzing the behavior of Gbx2-expressing cells in the diencephalon of wild-type and Gbx2-mutant embryos. Using a novel Gbx2-CreER-ires-Egfp knock-in mouse line to carry out inducible genetic fate-mapping study, we determine the cell fate of Gbx2-expressing cells in the diencephalon at different embryonic stages. Our data show that the Gbx2-expressing cells and their descendents contribute to the entire thalamic nuclear complex, but not structures that are derived from the pretectum epithalamus and prethalamus, demonstrating that the thalamus is a developmental compartment. We also show that Gbx2 is essential for maintaining the integrity of the boundaries surrounding the developing thalamus. Finally, we show that Gbx2 acts cell-nonautonomously in controlling the histogenesis and boundary formation of the thalamus.

MATERIALS AND METHODS

Generation of Gbx2CreER+ mouse line

A CreERT2-ires-Egfp-neo cassette was inserted into the 5’ untranslated region (UTR) of the Gbx2 locus by homologous recombination in mouse embryonic stem (ES) cells (Fig. 1A). From 63 G418 resistant ES cell colonies, seven correctly targeted ES cell clones were identified by Southern blot analysis (Fig. 1B). Germline chimeras were generated from two independent ES clones, which provided the identical phenotype. The neo cassette, which was flanked by two FRT sites, was removed in vivo by the hACTB-FLP transgene (Rodriguez et al., 2000). The new Gbx2 knock-in allele is designated as Gbx2CreER.

Mouse breeding and genotyping

Mice were maintained on an outbred CD1 genetic background (Charles River Lab, Wilmington, MA). Noon of the day on which the vaginal plug was found was designated as E0.5. For inducible genetic fate mapping, Gbx2CreER+; R26R−/− males, homozygous for the Cre reporter R26R...
(Soriano, 1999), were bred with wild-type or Gbx2+/– females (Wassarman et al., 1997). Four to six milligrams of tamoxifen (Sigma) in corn oil (20 mg/ml) was administered by oral gavage to pregnant females as described previously (Li and Joyner, 2001). Genotypes of mice were determined by PCR analysis. For PCR analysis of mosaic deletion, 200 μm brain slices were obtained by vibratome sectioning, and the thalamus, which is demarcated by EGFP, of Gbx2CreERF, R26R+/– embryos at E16.5 was dissected under a fluorescent stereoscope. The following primers were used to distinguish the floxed and deletion ES cells resulted in Gbx2-deficient ES cells (Gbx2–/–). To generate chimeras, Gbx2+/– or control Gbx2+/–/– ES cells were injected into blastocysts that carried the ROSA26 gene trap insertion, which expresses β-galactosidase (β-gal) ubiquitously (Friedrich and Soriano, 1991).

β-galactosidase, BrdU labeling, immunofluorescence and in situ hybridization

Embryos or brains were processed for in situ hybridization as described previously (Guo and Li, 2007). Standard X-gal staining was used to examine β-gal activities (Nagy et al., 2003). BrdU labeling was performed as described previously (Li et al., 2002). Pregnant females were injected intraperitoneally with 100 μg BrdU per gram of body weight 1.5 hours before they were sacrificed. Detailed protocols are available in the Li lab website (http://www.genetics.uchc.edu/lilab/Pages/Protocols.html). Antibodies used in the study are the following: rabbit anti-GFP (Invitrogen), mouse anti-BrdU (BD), mouse anti-TuJ1 (Covance) and Alexa fluorescent secondary antibodies (Invitrogen).

RESULTS

Generation of a knock-in mouse line with simultaneous expression of CreER and EGFP recapitulating endogenous Gbx2 expression

To gain more insight into the function of Gbx2, we generated a knock-in mouse line Gbx2CreER, in which CreER-ires-Egfp was targeted into the 5′ UTR of the Gbx2 gene (Fig. 1A). As the CreER-ires-Egfp cassette contains polyadenylation signals and stop codons, we expected that the insertion would result in a null mutation of Gbx2. Indeed, homozygous Gbx2CreER/CreER compound heterozygous Gbx2CreER/CreER mutants, which contain a previously characterized Gbx2-null allele, exhibited an identical phenotype to Gbx2–/– embryos (Miyashita-Lin et al., 1999, Wassarman et al., 1997) (Fig. 1C, D and see below).

In Gbx2CreER/+ embryos, CreER transcripts and EGFP proteins were detected in the same domain as the endogenous Gbx2 expression between E10.5 and E16.5 (Fig. 2A-C and data not shown). In the diencephalon of Gbx2CreER/+ embryos at E12.5, EGFP was detected in cells in the mantle zone and their axons, which traversed through the prethalamus toward ventral telencephalon (Fig. 2C, D, F, H). To determine if Gbx2-expressing cells in the diencephalon are postmitotic, we performed colocalization studies of EGFP and BrdU in Gbx2CreER/+ embryos with BrdU pulse (1.5 hour) labeling at E12.5. EGFP and BrdU signals were found largely mutually exclusive (Fig. 2D-E*). We also performed colocalization analysis of EGFP and the mitotic marker phosphorylated histone H3 (pH3) on serial coronal sections of the thalamus of E12.5 Gbx2CreER/+ embryos by confocal microscopy (Hendzel et al., 1997). At the rostral and caudal level, EGFP- and pH3-positive cells were largely segregated (data not shown). At the middle level, pH3 signals were detected in a broad domain outside the ventricular layer of the thalamus, with some positive cells embedding in the EGFP-positive domain (Fig. 2F). We did detect a few pH3-positive cells with weak GFP signals (Fig. 2G*). However, the majority of the pH3-positive cells were negative for EGFP. Furthermore, examination of a marker for postmitotic neuronal precursors, neuronal class III β-tubulin (TuJ1), revealed that EGFP was completely colocalized with TuJ1 in the diencephalon of Gbx2CreER/+ embryos at E11.5 (Fig. 2F-G*). These data demonstrate that Gbx2 is primarily expressed in the neuronal precursor cells that have exited the cell cycle in the diencephalon.

The Gbx2-expressing cells and their descendents form a self-contained compartment corresponding to the entire thalamus

To examine the fate of Gbx2-expressing cells in the diencephalon, we performed inducible genetic fate mapping by combining Gbx2CreER and the R26R reporter alleles (Soriano, 1999). In Gbx2CreER/+; R26R embryos, Cre-mediated recombination at the R26R locus in cells that express activated CreER will result in permanent expression of β-gal in these cells and all their descendents (Joyner and Zervas, 2006). Activation of CreER is achieved by administration of tamoxifen to pregnant females carrying Gbx2CreER/+; R26R embryos. We administered tamoxifen at E10.5, when Gbx2 is already expressed in the diencephalon (Nakagawa and O’Leary, 2001), and assessed embryos 24 hours later.
later to delineate the initially marked cohort of Gbx2-expressing cells. β-Gal-positive cells were detected in the diencephalon, anterior hindbrain and spinal cord of Gbx2CreER/+; R26R embryos (Fig. 3B; not shown). This pattern of β-gal expression was remarkably similar to that of Gbx2 expression at E10.75, demonstrating that the activation of CreER by tamoxifen faithfully labels Gbx2-expressing cells in Gbx2CreER/+; R26R embryos (Fig. 3A). No β-gal-positive cells were detected in Gbx2CreER/-; R26R embryos (n≥10) without tamoxifen administration, demonstrating that Cre activity from the Gbx2CreER allele is tamoxifen-dependent (see Fig. S1A in the supplementary material). In agreement with previous reports (Hayashi and McMahon, 2002; Zervas et al., 2004), we found that labeling of Gbx2-expressing cells in the diencephalon was largely restricted to a window of 6 to 36 hours after tamoxifen administration (see Fig. S1B-E in the supplementary material).

We next examined the fate of Gbx2-positive cells in the diencephalon marked at E10.5. Analysis of β-gal activity in whole-mount brains at E18.5 showed that marked cells were confined to the presumptive thalamus in Gbx2CreER/+ embryos (Fig. 3C). Histological analysis revealed that the fate-mapped cells contributed broadly to the thalamus and formed remarkably sharp boundaries delineating the thalamus from the neighboring brain structures (Fig. 3D-F). Examination of X-gal and Nissl staining on adjacent sections revealed that the anterior and posterior boundaries delineated by β-gal-positive cells strictly coincided with the histological borders demarcating the thalamus from the pretectum and the prethalamus (Fig. 4E,G). Furthermore, the β-gal-positive cells defined a clear dorsal border separating the thalamus from the epithalamus, with only a few marked cells in the lateral habenular nuclei (Fig. 4M,O). The ventral border of the thalamus was also clearly defined by the marked cells, except for some marked cells in the nucleus of Darkschewitsch (Fig. 4E), which is presumably derived from the basal plate of p1 and p2 (Puelles and Rubenstein, 2003).

The thalamic cells that express Gbx2 at different stages form distinct groups of thalamic nuclei

Previous data on Gbx2 expression in the developing thalamus between E10.5 and postnatal day 2 (P2) have suggested that Gbx2 is specifically expressed and maintained in subsets of thalamic neurons that form the anterior and medial groups of thalamic nuclei (Jones and Rubenstein, 2004; Nakagawa and O’Leary, 2001). Surprisingly, we found that the marked descendents of Gbx2-expressing cells labeled by tamoxifen administration at E10.5 broadly contribute to the thalamus in Gbx2CreER/+; R26R embryos (Fig. 3C-F). To resolve this apparent inconsistency, we sought to examine if Gbx2-expressing cells at other stages might have preferential contribution to particular thalamic nuclei.

As CreER is active within a window of 6-36 hours after the administration of tamoxifen, we gave tamoxifen at E9.5, and determined the contribution of the initial Gbx2-expressing thalamic cells in Gbx2CreER/+; R26R mice at P15, when various thalamic nuclei can be identified by Nissl histology (Caviness and Frost, 1980; Jones, 2007). The marked cells were found in the lateral-posterior and ventral thalamic nuclei groups (L, LGd, VM, VL, VB, Pom, VMb, LP and MG), but not the anterior and medial thalamic nuclei group (Fig. 5A-C; Table 1). The fate-mapped cells labeled at E10.5 were present in most of the thalamic nuclei in the caudal and lateral regions of the thalamus, whereas the rostromedial-most nuclei contained a dramatically reduced number of β-gal-positive cells (Fig. 5D-F; Table 1). When tamoxifen was administered at E15.5, β-gal-positive cells were found in the anterior (AD, AM and
Gbx2 is required for forming the dorsal and posterior, but not the anterior and ventral, borders of the thalamus

The remarkably sharp Gbx2-lineage borders led us to investigate if Gbx2 is required for the boundary formation by fate mapping Gbx2-transcribing cells in Gbx2CreER/–; R26R embryos, which lack Gbx2 function (Fig. 1C,D). When tamoxifen was administered to pregnant Gbx2+/− females that were mated with Gbx2CreER/–; R26R+ males at E10.5, the distribution of β-gal-positive cells in the diencephalon was indistinguishable between Gbx2CreER/–; R26R+ and Gbx2CreER/–; R26R embryos at E11.5 and E12.5 (Fig. 4A,B; data not shown). These results demonstrate that the initial transcription of the Gbx2 locus in thalamic precursors is independent of Gbx2 protein activity. This enabled us to examine whether the marked thalamic cells continued to be restricted to the thalamic compartment without Gbx2.

We found that the morphology of the thalamus in Gbx2CreER/+; R26R was severely disrupted after E14.5, similar to that found in Gbx2−/− mutants (Miyashita-Lin et al., 1999). The thalamus, which was demarcated by the marked descendents of Gbx2-transcribing cells labeled at E10.5, was apparently reduced in the mediolateral dimension but expanded in the ventrodorsal dimension, resulting in an abnormal shape (Fig. 4D,F,L,N). A large number of marked descendents of Gbx2-transcribing cells labeled at E10.5 were across the dorsal and posterior borders of the thalamus expanding into the epithalamus and the pretectum, respectively, in Gbx2CreER/–; R26R embryos at E14.5 (Fig. 4D, and data not shown). The marked cells that crossed the lineage border were mainly found in the lateral habenular nuclei (Fig. 4N,P) and the anterior part of the pretectum (Fig. 4F,L) in the mutants at E18.5. In contrast to the dorsal and the posterior borders, the anterior and ventral borders of the thalamus were much less affected, with the fate-mapped cells demarcating a clear thalamus-prethalamus boundary in Gbx2CreER/–; R26R embryos at E18.5 (Fig. 4F,N). In agreement with the cell-fate mapping data, histological analysis by Nissl staining revealed that the dorsal and posterior, but not the anterior and ventral, borders of the thalamus were disrupted in Gbx2CreER/–; R26R embryos (Fig. 4G-H,Q-R). Collectively, our data demonstrate that Gbx2 is required for the formation of the dorsal and posterior boundaries separating the thalamus from the epithalamus and the pretectum, respectively. However, a Gbx2-independent mechanism is involved in the development of the anterior and ventral boundaries of the thalamus.

Loss of Gbx2 does not result in a major patterning defect in the diencephalon

The severe disruption in the histogenesis and the dorsal and posterior borders of the thalamus in Gbx2 mutants prompted us to examine by marker analysis if Gbx2 is required for maintaining the fate of thalamic cells. At E12.5, the expression domains of Gbx2 and Dlx2/5 demarcate the thalamus and prethalamus, respectively, while Shh is expressed in the zona limitans intrathalamica (ZLI) at the interface between the thalamus and prethalamus (Bulfone et al., 1993) (Fig. 6A-D). In agreement with a previous study (Miyashita-Lin et al., 1999), the transcripts of truncated Gbx2 were detected in the same domain in the lateral wall of the diencephalon in Gbx2creER/– embryos at E12.5 as that found in wild-type embryos (Fig. 6A,E). Furthermore, in Gbx2creER/– embryos at E12.5, Dlx2/5 and Shh were each detected in the same domain as those in wild-type embryos (Fig. 6E-H). Lhx1, Pax3 and Pax7 are expressed in the pretectum at E13.5 (Fig. 6I,J; and data not shown). In addition, Lhx1 is also expressed in the ZLI (Fig. 6I). Again, no difference in Lhx1, Pax3 and Pax7 expression was observed in Gbx2−/– embryos (Fig. 6L,M; data not shown). Examination of another pretectum marker, Bhlhb4, which encodes a basic helix-loop-helix transcription factor (Bramblett et al., 2002), showed that its expression was also normally restricted to the anterior pretectum in Gbx2−/– embryos at E13.5 as in wild type (Fig. 6K,N). Together, our data suggest that the abnormal histogenesis and the disruption of thalamic boundaries in Gbx2 mutants do not result from obvious defects in patterning or cell-fate specification in the diencephalon.

Gbx2 plays a cell-nonautonomous function in the formation of thalamic boundaries

Differential affinities for cell-cell interactions have been proposed as a basic mechanism for separating cells into distinct compartments (Irving and Rauskolb, 2001; Kiecker and Lumsden, 2005). Indeed, members of the Cadherin family and other cell adhesion molecules are expressed in stripes or patches in the diencephalon with their expression coinciding with prosomeric borders or developing thalamic nuclei (Gao et al., 1998; Mackarehtschian et al., 1999; Redies et al., 2000; Yoon et al., 2000). We therefore probed the possibility that Gbx2 controls...
chimeric embryos. Chimeric embryos were generated by injecting Gbx2+/– or Gbx2–/– ES cells into the blastocysts that were heterozygous for the ubiquitous ROSA26 lacZ reporter allele so that the host cells could be identified by β-gal activity (Friedrich and Soriano, 1991). Surprisingly, we found that Gbx2–/–, like Gbx2+/– cells, were present throughout the thalamus and intermingled with the host cells in the thalamus of the chimeric embryos at E16.5 (Fig. 7E-H). By contrast, the mutant cells were segregated from the wild-type cells and aggregated specifically in the cerebellum of the chimeric embryos (Fig. 7F). These observations demonstrate that there are distinct cellular requirements for Gbx2 in the thalamus and the cerebellum. In the cerebellum, Gbx2 appears to act cell-autonomously in regulating cell adhesion. However, in the thalamus, the normal cell mixing suggests that wild-type and mutant cells have similar cell-adhesive properties. Alternatively, wild-type cells may rescue the defect of cell adhesion of Gbx2–/– cells in the thalamus. Consistent with a possible cell-nonautonomous role of Gbx2, we found that, in sharp contrast to those found in Gbx2-null mutants, the morphology and the histological borders of the thalamus were remarkably normal in the chimeras that were composed of Gbx2–/– and wild-type cells (Fig. 7F).

We next investigated whether the mutant thalamic neurons are prevented from dispersing into the epithalamus or the pretectum in chimeric embryos. We performed genetic mosaic analysis by thalamic boundaries by regulating expression of these cell surface molecules. On serial coronal sections of E14.5 brain, Cdh6 expression was detected in the medial part of the thalamus, and its expression domain becomes a narrow band in the lateral region with its dorsal and posterior limits clearly delineating the thalamus from the epithalamus and the pretectum, respectively (Fig. 7A; data not shown). In Gbx2 mutants at E14.5, the dorsal border of Cdh6 expression was indiscernible, although diffuse expression of Cdh6 persisted in the presumptive thalamus (Fig. 7C). Efna5 encodes a member of the EphrinA ligand family. By interacting with EphA receptors, EphrinA ligands mediate cell segregation in rhombomeres of vertebrate hindbrains (Xu et al., 1999). In the E14.5 diencephalon, Efna5 is expressed in four transverse stripes flanking the p1-2 and p2-3 borders, respectively (Fig. 7B). Without Gbx2, the expression of Efna5 in the thalamus was lost, whereas the two transverse bands of Efna5 expression in the pretectum and the prethalamus were unaffected (Fig. 7D). These data appear to be consistent with a possible role of Gbx2 in regulating cell adhesive properties of thalamic neurons.

To determine if loss of Gbx2 indeed alters cell adhesion in the thalamus, we performed chimera experiments. We reasoned that an alteration in cell-adhesive properties due to loss of Gbx2 would lead to abnormal mixing of the Gbx2-mutant and wild-type cells in chimeric embryos. Chimeric embryos were generated by injecting Gbx2+/+ or Gbx2−/− ES cells into the blastocysts that were heterozygous for the ubiquitous ROSA26 lacZ reporter allele so that the host cells could be identified by β-gal activity (Friedrich and Soriano, 1991). Surprisingly, we found that Gbx2−/−, like Gbx2+/+ cells, were present throughout the thalamus and intermingled with the host cells in the thalamus of the chimeric embryos at E16.5 (Fig. 7E-H). By contrast, the mutant cells were segregated from the wild-type cells and aggregated specifically in the cerebellum of the chimeric embryos (Fig. 7F). These observations demonstrate that there are distinct cellular requirements for Gbx2 in the thalamus and the cerebellum. In the cerebellum, Gbx2 appears to act cell-autonomously in regulating cell adhesion. However, in the thalamus, the normal cell mixing suggests that wild-type and mutant cells have similar cell-adhesive properties. Alternatively, wild-type cells may rescue the defect of cell adhesion of Gbx2−/− cells in the thalamus. Consistent with a possible cell-nonautonomous role of Gbx2, we found that, in sharp contrast to those found in Gbx2-null mutants, the morphology and the histological borders of the thalamus were remarkably normal in the chimeras that were composed of Gbx2−/− and wild-type cells (Fig. 7F).
and E18.5, similar to those found in Gbx2CreER/+; R26R embryos (compare Fig. 7K, inset in Fig. 7O with Fig. 3E,F; Fig. 4E,F, and Fig. 4M-P). Mosaic embryos that contained stronger β-gal activity in the thalamus did exhibit a mild defect in the morphology of the thalamus (n=7; Fig. 7L). These results suggest that in the presence of wild-type cells, the dorsal and posterior boundaries of the thalamus are rescued in the genetic mosaic embryos.

Finally, we examined if the expression of Efna5 is rescued in the presence of wild-type cells in the thalamus of chimeric and mosaic embryos. In contrast to that in Gbx2-null mutants, Efna5 is expressed at the dorsal and the caudal borders of the thalamus in both chimeric or mosaic embryos at E16.5 (Fig. 7M-P). Efna5 expression was expanded in the chimeric and mosaic embryos. Taken together, the results of our chimeric and genetic mosaic analysis demonstrate that Gbx2 proteins function cell-nonautonomously in controlling the histogenesis and the boundary formation of the thalamus.

Fig. 5. Descendents of Gbx2-expressing cells at different developmental stages populate distinct thalamic nuclei. (A-I) X-gal staining of coronal sections at the rostral, middle and caudal levels of the thalamus of Gbx2CreER/+; R26R mice at P15 after administration of tamoxifen at E9.5 (A-C), E10.5 (D-F) and E15.5 (G-I). The asterisk indicates some marked cells outside the medial geniculate nucleus. The arrowheads indicate marked cells that originate from Gbx2-expressing cells in the medial ganglionic eminence, in the caudateputamen and globus pallidus. (J-L) Schematic summary of five classes of thalamic nuclei formed by temporally distinct Gbx2-expressing cells. The nuclei marked by light blue, light green and red represent nuclei formed by the initial wave (between E9.5 and E10.5), second wave (between E10.5 and E11.5) and the final wave (E15.5) of Gbx2-expressing cells, respectively. The nuclei in which Gbx2 expression is maintained to postnatal stages are indicated by blue dots. CP, caudateputamen; GP, globus pallidus; HyT, hypothalamus; MGE, medial ganglionic eminence; SC, superior colliculus. See Table 1 for abbreviations of thalamic nuclei. Scale bar: 400 μm.

Fig. 6. Loss of Gbx2 does not result in obvious defects in patterning of the diencephalon. (A-H) In situ hybridization assay on coronal sections of Gbx2CreER/+ (A-D) and Gbx2CreER/- embryos (E-H) at E12.5 with different markers for the thalamus, pretalamus and ZLI as indicated. (I-N) Analysis of markers as indicated for the pretectum on sagittal sections of Gbx2CreER/+ (I-K) and Gbx2CreER/- embryos (L-N) at E13.5. The border between the thalamus and the pretectum is demarcated by the cell-free zone, corresponding to the habenulopeduncular tract. The arrowheads indicate the border between the epithalamus and the thalamus; the arrows mark the ZLI. ET, epithalamus; HPT, habenulopeduncular tract; NCx, neocortex; PT, pretectum; PTh, pretalamus; TH, thalamus. Scale bar: 220 μm in A-H; 200 μm in I-N.

combining the Gbx2CreER allele with a conditional Gbx2 deletion allele, Gbx2- (Li et al., 2002). Taking advantage of the mosaic manner of CreER-mediated recombination (Joyner and Zervas, 2006), we expected that administration of tamoxifen at E10.5 would produce a genetically mosaic thalamus composed of Gbx2CreER/F (Gbx2 heterozygous – wild type in phenotype) and Gbx2CreER/- (Gbx2 null) cells in Gbx2CreER/F; R26R embryos, whereas the Gbx2CreER/- cells would be probably marked by β-gal (Fig. 7I). PCR analysis of microdissected thalamic tissues showed that the administration of tamoxifen at E10.5 indeed produced a genetically mosaic thalamus composed of Gbx2CreER/F and Gbx2CreER/- cells in Gbx2CreER/F; R26R embryos at E16.5 (Fig. 7J). Significantly, the morphology of the thalamus was largely normal in the genetic mosaic embryos that contained a significant number of β-gal-positive cells in the thalamus (n=11) (Fig. 7K and inset in Fig. 7O). The labeled descendents of Gbx2-expressing cells were restricted to the thalamic compartment in Gbx2CreER/F; R26R embryos at E14.5 and E18.5, similar to those found in Gbx2CreER/+; R26R embryos (compare Fig. 7K, inset in Fig. 7O with Fig. 3E,F; Fig. 4E,F, and Fig. 4M-P). Mosaic embryos that contained stronger β-gal activity in the thalamus did exhibit a mild defect in the morphology of the thalamus in Gbx2CreER/- embryos (n=7; Fig. 7L). These results suggest that in the presence of wild-type cells, the dorsal and posterior boundaries of the thalamus are rescued in the genetic mosaic embryos. Finally, we examined if the expression of Efna5 is rescued in the presence of wild-type cells in the thalamus of chimeric and mosaic embryos. In contrast to that in Gbx2-null mutants, Efna5 is expressed at the dorsal and the caudal borders of the thalamus in both chimeric or mosaic embryos at E16.5 (Fig. 7M-P). Interestingly, the expression domains of Efna5 appear to be expanded in the chimeric and mosaic embryos. Taken together, the results of our chimeric and genetic mosaic analysis demonstrate that Gbx2 proteins function cell-nonautonomously in controlling the histogenesis and the boundary formation of the thalamus.
the entire thalamus. Significantly, the fate-mapped compartment, which is defined by the expression domain of thus revealed the presence of a hitherto unknown developmental
formation in the thalamus. The developmental compartment defined by the Gbx2 lineage contrasts with the known compartments in the vertebrate hindbrain and telencephalon, where the postmitotic cells in the mantle zone are known to be able to cross rhombomeric or the pallial-subpallial boundaries, although their progenitors in the proliferating zone are restricted to a cell-tight compartment (Fishell et al., 1993; Wingate and Lumsden, 1996). It has been postulated that compartmental boundaries are mainly required for a proliferating cell population with labile cell fates, whereas boundary restriction becomes dispensable for postmitotic cells, as their fates are specified (Kiecker and Lumsden, 2005). Therefore, the confinement of the Gbx2-expressing cells and their descendents, which are mainly postmitotic, within the thalamic compartment may serve a different function from those compartments. Interestingly, we observed that the borders of the Gbx2 lineage marked at E10.5 were progressively sharpened between E14.5 and E16.5 (see Figs 3 and 4), coinciding with the initial parceling of the dorsal thalamus (Jones, 2007). We speculate that the lineage restriction of postmitotic Gbx2-positive thalamic cells may underlie the formation of thalamic nuclei. By fate mapping Gbx2-expressing cells at E9.5, E10.5 or E15.5, we have identified five groups of the thalamic nuclei (summary in Fig. 5J-L). The initial Gbx2-expressing cells (around E10.5) give rise to most of the principal relay nuclei, such as LGd, VB, LP and MG. However, Gbx2

| Anterior group | Anterior dorsal nucleus | AD | – | ++ | ++ |
| Medial group   | Medial dorsal nucleus   | MD | + | ++ | +++ |
| Intralaminar group | Central medial nucleus | Ce | + | ++ | +++ |
| Ventrual nuclei | Ventrabraasal nucleus   | VB | ++ | +++ | – |
| Posterior group | Lateral posterior nucleus | LP | ++ | +++ | +++ |

+++ large numbers of labeled cells.
++, moderate number of labeled cells.
+, a few cells.
–, none or few labeled cells.

Nomenclature and classification of thalamic nuclei follow Caviness and Frost (Caviness and Frost, 1980), and Jones (Jones, 2007).

**DISCUSSION**

Gbx2-expressing cells and their descendents define the lineage-restriction boundaries of the thalamus

The prosomeric model proposed by Puelles and Rubenstein has provided us with an important conceptual framework for understanding the development of the forebrain (Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). However, it remains controversial whether the prosomeres represent true development compartments that are units of cell lineage restriction similar to rhombomeres in the vertebrate hindbrain (Figdor and Stern, 1993; Larsen et al., 2001; Zelser et al., 2001). According to the prosomeric model, the thalamus is generated from the alar plate with labile cell fates, whereas boundary restriction becomes dispensable for postmitotic cells, as their fates are specified (Kiecker and Lumsden, 2005). Therefore, the confinement of the Gbx2-expressing cells and their descendents, which are mainly postmitotic, within the thalamic compartment may serve a different function from those compartments. Interestingly, we observed that the borders of the Gbx2 lineage marked at E10.5 were progressively sharpened between E14.5 and E16.5 (see Figs 3 and 4), coinciding with the initial parceling of the dorsal thalamus (Jones, 2007). We speculate that the lineage restriction of postmitotic Gbx2-positive thalamic cells may underlie the formation of thalamic nuclei. By fate mapping Gbx2-expressing cells at E9.5, E10.5 or E15.5, we have identified five groups of the thalamic nuclei (summary in Fig. 5J-L). The initial Gbx2-expressing cells (around E10.5) give rise to most of the principal relay nuclei, such as LGd, VB, LP and MG. However, Gbx2
expression is downregulated in LGd and VB (designated as group I nuclei) after E10.5, and persists in LP and MG (group II). The second wave of Gbx2-expressing cells (E10.5-E11.5) gives rise to many association nuclei, such as AV, L, C1, MD, PC, Ce, VMb and Re, and relay nucleus L. Among these nuclei, Gbx2 expression is maintained in MD and CI, PC and Ce (group III), and lost in L and VMb (group IV). The last wave of Gbx2-expressing cells (E15.5) gives rise to the most anteromedial nuclei, AD, AM, PV and Re (group V), where Gbx2 expression persists into postnatal stages. Therefore, the precursors for distinct groups of thalamic nuclei display dynamic and distinct temporal patterns of Gbx2 expression, although all thalamic neurons are derived from the Gbx2 lineage. These observations suggest that the expression of Gbx2 itself allows the thalamus as a whole to be segregated from the neighboring structures, which never express Gbx2. Within the thalamus, however, the dynamic and differential expression of Gbx2 may lead to segregation of Gbx2-positive neurons from Gbx2-negative neurons, which have not yet started or have lost Gbx2 expression.

**Specific requirements for Gbx2 in the formation of the dorsal and caudal lineage boundaries of the thalamus**

Birth-dating analysis using [3H]-thymidine autoradiography has previously demonstrated an ‘outside-in’ gradient, with earlier-born neurons being displaced outside by later-born neurons in the diencephalon (Angevine, 1970). This arrangement of thalamic neurons according to their time of origin raises the question of whether the cell-tight thalamic compartment revealed by the current study is simply a result of lack of cell movement in the developing thalamus, however, the dynamic and differential expression of Gbx2 may lead to segregation of Gbx2-positive neurons from Gbx2-negative neurons, which have not yet started or have lost Gbx2 expression.

**Fig. 7. Gbx2 plays a cell-nonautonomous role in controlling the thalamic lineage boundaries.** (A-D) In situ hybridization analysis of Cdh6 and Efna5 expression on the coronal brain sections of Gbx2CreER/+, Gbx2CreER–/– embryos at E14.5. The arrowhead indicates the expression in the thalamus; the asterisk marks the diminished Cdh6 and lost Efna5 expression in the mutant embryos. (E-H) X-gal and Fast Red staining of sagittal brain sections of E16.5 chimeric embryos composed of wild-type (blue) and ES-derived cells (pink) of genotype Gbx2CreER+/– (E) or Gbx2CreER–/– (F). The arrow indicates aggregates of Gbx2CreER–/– cells in the cerebellum; the arrowheads indicate the sharp thalamic borders. (G,H) Magnifed view of the thalamus in E and F. Note that the Gbx2CreER–/– cells, like Gbx2CreER+/–, extensively intermingle with the host cells in the thalamus. (I) Schematic diagram illustrating the generation of genetic mosaics using CreER-mediated deletion of Gbx2 in the thalamus of Gbx2CreER+/–, R26R embryos. The arrows indicate the primers for PCR analysis to detect the floxed (1.7 kb) and deletion (0.4 kb) alleles of Gbx2. (J) PCR analysis of microdissected thalamus of Gbx2CreER+/– and Gbx2CreER–/– cells after tamoxifen administration, but only Gbx2CreER+/– cells without tamoxifen. (K,L) X-gal staining of coronal brain sections of two Gbx2CreER+/–, R26R embryos with different levels of β-gal activity at E18.5 after tamoxifen administration at E10.5. The arrowheads mark the sharp borders of the fate-mapped Gbx2 lineage. (M-P) In situ hybridization of Efna5 on sagittal brain sections of Gbx2CreER+/– (M), Gbx2CreER–/– wild-type chimera (N), Gbx2CreER+/– Gbx2CreER–/– mosaic (O), and Gbx2CreER–/– (P) embryos at E16.5. Inset in O shows X-gal staining of a sagittal section of the mosaic embryo. The arrows indicate the restored expression of Efna5 in the chimeric and mosaic embryos; the asterisk indicates the absence of Efna5 expression in the thalamus of Gbx2CreER–/– embryo. ET, epithalamus; Ncx, neocortex; PT, pretectum; PTh, prethalamus; TH, thalamus; WT, wild type. Scale bars: 400 μm in A-D; 450 μm in E,F; 29 μm in G,H; 380 μm in K; 200 μm in L-O.
Gbx2 function in thalamic development

A cell-nonautonomous role of Gbx2 in the regulation of lineage-restriction boundary of the thalamus

Given that the expression of Cdh6 and Efna5 is disrupted in the thalamus of Gbx2 mutant embryos, we were surprised to discover that Gbx2-deficient and wild-type cells intermix normally in the thalamus of chimeric and genetic mosaic embryos. Our data strongly suggest that Gbx2 plays a cell-nonautonomous role in the formation of the thalamic boundaries. First, we found that the morphology and the histological border of the thalamus are remarkably normal in the chimeric and mosaic embryos that contain a significant percentage (not less than 50%) of Gbx2-deficient cells, as judged by PCR and β-gal expression. It is remarkable that mosaic embryos that contained strong β-gal activity in the thalamus did exhibit a mild defect in the morphology of the thalamus in Gbx2CreER/F; R26R embryos. These results indicate that administration of tamoxifen indeed leads to deletion of Gbx2, and deletion of Gbx2 after E10.5 can still recapitulate the defect of Gbx2-null cells. Therefore, the rescue observed in the mosaic embryos is unlikely to be due to the residual Gbx2 proteins produced before CreER-mediated deletion occurs. The mild phenotype in the mosaic embryo with strong β-gal activity also suggests that a certain percentage of wild-type cells may be required for the rescue of the mutant phenotype. Second, in Gbx2Δ2-; CreER/F; R26R embryos with mosaic deletion of Gbx2 at E10.5, the marked descendents of Gbx2-expressing cells become normally restricted to the thalamic compartment. It is reasonable to assume that a significant number of the marked cells in Gbx2CreER/F; R26R embryos have lost Gbx2 due to CreER-mediated recombination. The absence of β-gal-positive cells in either the epithalamus or the pretectum demonstrates that the presence of wild-type cells rescues the lineage-restriction boundaries of the thalamus in mosaic Gbx2CreER/F; R26R embryos. We did not detect a bias of the wild-type cells being at the boundaries of the thalamus in chimeric embryos, arguing against the possibility that the wild-type cells may form border cells to restore the boundary. Finally, we found that Efna5 expression is restored in the thalamus of chimeric and mosaic embryos at E16.5. Because of the unavailability of suitable antibodies, we were unable to determine whether Efna5 is expressed in Gbx2-deficient cells in the chimeric or mosaic embryos. Nevertheless, the restored expression of Efna5 demonstrates that the dorsal and caudal borders of the thalamus are rescued in the chimeric and mosaic embryos. Collectively, our data demonstrate that Gbx2 acts cell-nonautonomously in regulating formation in the thalamic boundary. As Gbx2 is a transcription factor and presumably acts within the thalamic cells, we postulate that Gbx2 may regulate an extracellular signaling pathway, which in turn mediates the cell-nonautonomous role of Gbx2 in controlling boundary formation in the thalamus.

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Supplementary material

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References


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